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Molecular Epidemiology of Pneumococcal Carriage among Children with Upper Respiratory Tract Infections in Hanoi, Vietnam

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To investigate the molecular epidemiology of pneumococcal nasopharyngeal carriage in Hanoi, Vietnam, we studied 84 pneumococcal strains retrieved from children with upper respiratory tract infections. Serotypes 23F (32%), 19F (21%), 6B (13%), and 14 (10%) were found most often. A significant number of strains were antibiotic resistant. Fifty-two percent of the strains were (intermediate) resistant to penicillin, 87% were (intermediate) resistant to co-trimoxazole, 76% were resistant to tetracycline, 73% were resistant to erythromycin, and 39% were (intermediate) resistant to cefotaxime. Seventy-five percent were resistant to three or more classes of antibiotics. A high degree of genetic heterogeneity among the penicillin resistance genes was observed. In addition, the tetracycline resistance gene *tet*(M) and the erythromycin resistance gene *erm*(B) were predominantly observed among the isolates. Molecular analysis of the 84 isolates by restriction fragment end labeling (RFEL) revealed 35 distinct genotypes. Twelve of these genotypes represented a total of eight genetic clusters with 61 isolates (73%). The two largest clusters contained 24 and 12 isolates, and the isolates in those clusters were identical to the two internationally spreading multidrug-resistant clones Spain 23F-1 and Taiwan 19F-14, respectively. The remaining RFEL types were Vietnam specific, as they did not match the types in our reference collection of 193 distinct RFEL types from 16 countries. Furthermore, 57 of the 61 horizontally spreading isolates (93%) in the eight genetic clusters were covered by the seven-valent conjugate vaccine, whereas this vaccine covered only 43% of the isolates with unique genotypes. According to the serotype distribution of the nasopharyngeal pneumococcal isolates, this study suggests a high potential benefit of the seven-valent pneumococcal conjugate vaccine for children in Hanoi.

Streptococcus pneumoniae is one of the major causes of respiratory tract infections and invasive diseases in children all over the world. At present, worldwide about 1 million children under 5 years of age annually die of pneumococcal disease (18).

Pneumococci are often part of the nasopharyngeal flora; especially due to circumstances of crowding, as observed in day-care centers, nursing homes, and hospitals, the risk of being colonized with pneumococci is increased (3, 20, 27, 28). Usually, colonization is not followed by disease, as local barriers at the mucosal level of the respiratory tract and the human immune system are often protective. However, the balance between host and pathogen may be disturbed by highly pathogenic pneumococcal strains or by diminished host defense through viral infections, malnutrition, or immune deficiency (25).

Another problem is the growing (multi)drug resistance among pneumococcal isolates. The emergence of penicillin- and multidrug-resistant pneumococci has been observed in various countries over the last decade. In some countries and populations, up to 60% of the pneumococcal isolates are resistant to one or more antibiotics (1, 12, 22). A significant proportion of pneumococcal resistance is the result of the worldwide spread of a limited number of multidrug-resistant clones (6, 15, 35, 39).

Prevention of pneumococcal disease has become a major topic in the battle against pneumococcal disease. The prevailing 23-valent pneumococcal polysaccharide vaccines have been shown to be immunogenic in adults but not in the group most at risk of developing pneumococcal diseases, namely, young children (5). Recently, several conjugate vaccines have been developed which have proven to be effective in young children, especially against invasive diseases (2, 11). Unfortunately, these vaccines are protective against only a limited number of pneumococcal serotypes. Conjugate vaccines from up to 11 capsular serotypes have been developed, whereas over 90 serotypes exist. In order to evaluate the theoretical coverage of the vaccines and the effect of vaccination on future serotype distribution, we need to monitor in detail and on a large scale the molecular epidemiology of pneumococcal colonization and infection before and after the implementation of these vaccines.

So far, the coverage of the conjugate vaccine has been investigated in several parts of the world. For instance, approximately 75% of all pneumococcal central nervous system infections in children in Europe, the United States, and Canada are covered by the seven-valent conjugate vaccine. For China and Latin America, the rates are 50 and 48%, respectively (14). Several molecular epidemiological studies have been performed in Asian countries such as Thailand and Taiwan; however, in most studies the serotype distribution has not been investigated (8, 34). In South Korea the seven-valent conjugate

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vaccine covered 65% of the isolates retrieved in the period from 1991 to 1993 (22). Recently, the Asian Network for Surveillance of Resistant Pathogens has studied nasal carriage of pneumococci in healthy children in Taiwan, South Korea, Sri Lanka, and Vietnam. The most common serogroups were 6, 23, 19, 14, and 15. Because no subtypes were determined, the exact vaccine coverage could not be calculated (23).

In this study, we investigated the molecular epidemiology of 84 pneumococcal carriage isolates of children attending several outpatient departments in Hanoi, Vietnam, with acute respiratory tract infections. The serotype distribution, vaccine coverage rates, resistance patterns, and genetic properties of these isolates will be discussed.

MATERIALS AND METHODS

Serotyping. Eighty-four *S. pneumoniae* strains were isolated from the nasopharynges of 410 children with upper respiratory tract infections visiting the outpatient departments of the Vietnam-Cuba Clinic and the Children's and Bach Mai Hospitals in Hanoi from September 1997 to December 1999. Bacteriological diagnosis was performed according to procedures published in the *Manual of Clinical Microbiology* (11a), and pneumococci were serotyped by the capsular swelling method (Quellung reaction) with capular antisera (Statens Seruminstitut, Copenhagen, Denmark).

Susceptibility testing. The susceptibilities of the Vietnam strains to penicillin, co-trimoxazole, tetracycline, erythromycin, rifampin, vancomycin, ciprofloxacin, and cefotaxime were determined by the agar dilution method as described previously (13). To discriminate between susceptible and nonsusceptible strains, we used the antibiotic breakpoints according to the NCCLS guidelines (26).

Penicillin-binding protein (PBP) genotyping. The genetic polymorphisms of penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* of the pneumococcal isolates were investigated by restriction fragment length polymorphism (RFLP) analysis of the amplified genes, as described previously (4).

Detection and analysis of *erm*(B) and *mef* genes. To detect the presence of *erm*(B) in the pneumococcal isolates, we used the protocol described by Sutcliffe et al. (38). In summary, we amplified the genes by PCR and analyzed the amplified DNA products by agarose gel electrophoresis. The presence of the *mef* gene was also detected by PCR (38). In order to discriminate between *mef*(A) and *mef*(E), PCR-RFLP analysis was performed as described by Del Grosso et al. (9). The amplicon was restricted with *Bam*HI and *Dra*I. The *mef*(A) gene contains a single *Bam*HI site, which is absent in the *mef*(E) gene. Restriction of *mef*(A) and *mef*(E) with *Dra*I yields two and three fragments, respectively.

Detection of *tet*(M) and *tet*(O) genes. In order to discriminate between *tet*(M) and *tet*(O), PCR-RFLP analysis was performed. Primers tetOfw (5′-TGTCGG GTTGTCCATAGAG-3′) and tetOrev (5′-AAATTTACCAATAGCTGGC-3′) were used to amplify *tet*(O). The primer sequences were based on the *tet*(O) gene (GenBank accession number Y07780; positions 1455 to 1474 and positions 1957 to 1976, respectively). Primers tetM-fw (5′-CCATTGGTTTATCTGTATCA-3′) and tetM-rev (5′-CAGGTTCACCGGTAGTAACA-3′) were used to amplify *tet*(M). The primer sequences were based on the *tet*(M) gene (GenBank accession number X90939; positions 3428 to 3447 and 3930 to 3949, respectively). The PCR mixture consisted of 25 μl of reaction buffer containing 0.5 U of thermostable DNA polymerase diluted in the buffer supplied by the manufacturer (Integro, Leuvenheim, The Netherlands), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 pmol of each primer, and 10 to 50 ng of pneumococcal DNA. Amplification cycling in a programmable thermal controller (PTC-100; MJ Research, Watertown, Mass.) consisted of the following steps: predenaturation for 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. Amplification was finished after 3 min at 72°C. A 1.5% agarose gel in 0.5× TBE (Tris-borate-EDTA) containing 0.1 μg of ethidium bromide per ml was used to visualize the PCR products.

RFEL analysis. The 84 pneumococcal isolates were subjected to DNA fingerprinting analysis. Genotyping of the pneumococcal strains by restriction fragment end labeling (RFEL) analysis was performed as described by Van Steenbergen et al. (40) and adapted by Hermans et al. (16). Briefly, purified pneumococcal DNA was digested with restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-^{32}P]dATP by using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for various lengths of time at room temperature to ECL Hyperfilms (Amersham, Little Chalfont, United Kingdom).

Computer-assisted analysis of RFEL banding patterns. The RFEL types were analyzed by using the Windows version of GelCompar software (version 4; Applied Maths, Kortrijk, Belgium) after the RFEL autoradiograms were imaged with an Image Master desk top scanner (Pharmacia Biotech, Uppsala, Sweden). To this end, the DNA fragments in the molecular size range of 160 to 400 bp were explored. The DNA banding patterns were normalized with the pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages (30) and by using the Jaccard similarity coefficient applied to peaks (37). Computer-assisted analysis was carried out, and the methods were performed and the algorithms were used according to the instructions of the manufacturer of GelCompar. A tolerance of 1.2% in band position was applied during comparison of the DNA patterns. For evaluation of the genetic relatedness of the isolates, we used the following definitions: (i) strains of a particular RFEL type are 100% identical by RFEL analysis, (ii) an RFEL cluster represents a group of strains with RFEL types that differ by only one band (>95% genetic relatedness), and (iii) an RFEL lineage represents a group of strains with RFEL types that differ by less than four bands (>85% genetic relatedness).

International comparison. The Vietnamese genotypes were compared with the genotypes of an international collection of pneumococcal strains representing 193 distinct RFEL types originating from 15 different countries in Europe, Africa, and Asia and from the United States (M. Sluijter, unpublished observations, 2002), in which all international pandemic clones described by the pneumococcal epidemiological network are present (http://www.wits.ac.za/pmen/pmen.htm).

RESULTS

Eighty-four isolates retrieved from the nasopharynges of children under 5 years of age with acute respiratory tract infections were investigated by serotyping, susceptibility testing, and RFEL analysis. The most frequently observed serotypes were 23F (32% of strains), 19F (21%), 6B (13%), and 14 (10%). The rate of coverage of the seven-valent conjugate vaccine (covering serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was 80%. The additive rate of coverage of the 11-valent conjugate vaccine (covering the additional serotypes 1, 3, 5, and 7F) was negligible: only one serotype 3 isolate was found, and isolates of the other three serotypes were absent. The most common serotypes not covered by the conjugate vaccine were 23A (2%), 6A (4%), and 15 (4%); one isolate could not be serotyped.

The rate of resistance to one or more antibiotics was 96%. The three isolates susceptible to all antibiotics were nonvaccine capsular types. The proportions of strains resistant to penicillin, co-trimoxazole, tetracycline, and erythromycin were 39, 71, 76, and 73%, respectively. None of the strains were resistant to rifampin, vancomycin, or ciprofloxacin. Intermediate resistance to penicillin, co-trimoxazole, and cefotaxime was found in 13, 16, and 39% of the strains, respectively. In total, 78% of the strains were multidrug resistant. These multidrug-resistant strains were of serotypes 23F (24 strains), 19F (17 strains), 6B (12 strains), 14 (6 strains), 9V and 16 (2 strains each), and 15, 23A, and untypeable (1 strain each).

Molecular analysis of the 84 isolates revealed 35 different genotypes. Twelve of these genotypes represented eight genetic clusters with 61 isolates (72%) (Fig. 1). The largest cluster, cluster VIII, consisted of 24 isolates of serotype 23F and RFEL type 015. The second largest cluster was cluster VI, which consisted of 12 isolates of serotype 19F and RFEL type 006. The third and fourth largest clusters had 10 and 7 isolates, respectively, and each cluster had two different genotypes. In

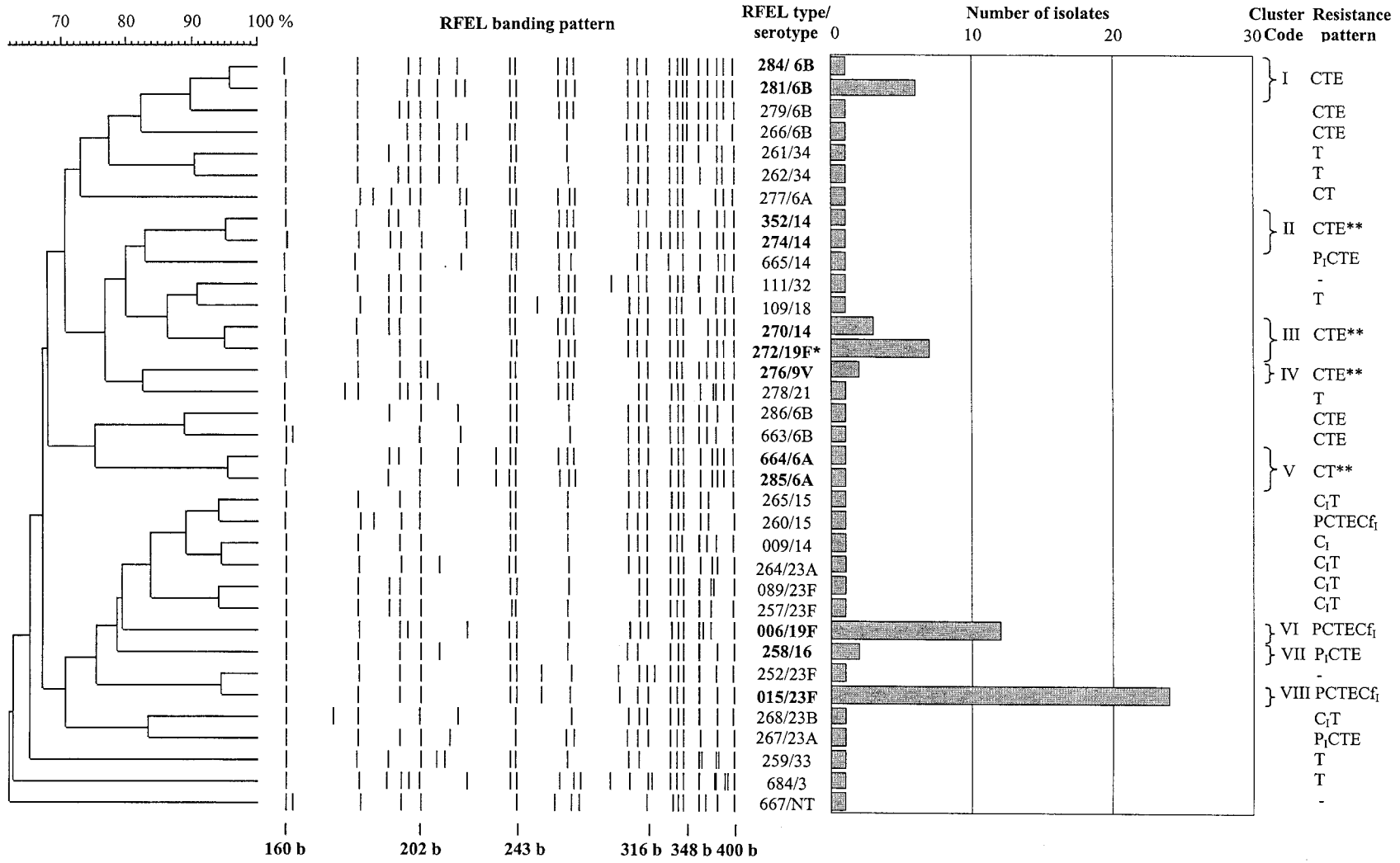

FIG. 1. Dendrogram of the 38 RFEL types observed among the 84 strains isolated from the nasopharynges of Vietnamese children with upper respiratory tract infections. The molecular sizes of reference bands (in bases [b] [bold]), RFEL banding patterns, RFEL types, serotypes, cluster codes, and resistance patterns are depicted. P, penicillin; C, co-trimoxazole; T, tetracycline; E, erythromycin; Cf, cefotaxime; I, intermediate. Bars represent the number of isolates per RFEL type. *, predominant serotypes within RFEL types; **, major resistance patterns observed within clusters.

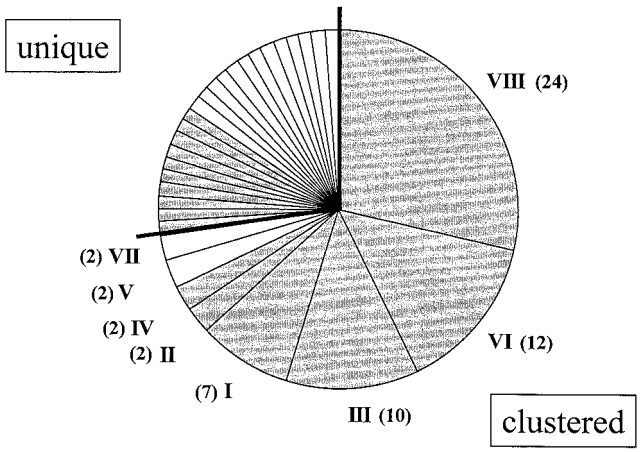

FIG. 2. RFEL genotypes and theoretical rates of coverage of the seven-valent conjugate vaccine. Vaccine serotypes are depicted in grey. Cluster codes and the number of isolates per cluster (in parentheses) are depicted.

contrast to all other clusters, cluster III, with 10 isolates, harbored isolates with two distinct capsular types, namely, serotypes 19F and 14. Cluster I, with seven isolates, consisted of serotype 6B strains only (Fig. 1).

Ninety-three percent of the isolates in six of the eight clusters were covered by the seven-valent conjugate vaccine, whereas only 43% of the isolates with unique genotypes were covered by the vaccine (Fig. 2).

We compared all Vietnamese RFEL types with the types in our international database representing 193 isolates from 16 different countries as well as those of all international pandemic clones described by the Pneumococcal Epidemiology Network (http://www.wits.ac.za/pmen/pmen.htm). Two of the genotypes, comprising the two largest clusters, clusters VIII and VI, were identical to international clones Spain 23F-1 and Taiwan 19F-14, respectively. The remaining 33 RFEL types were Vietnam specific, as these genotypes were not found in our present database.

PBP genotyping of the 84 pneumococcal isolates revealed 19 distinct types, and the cluster sizes ranged from 1 to 24 strains. Despite the observation that 61% of the strains were penicillin susceptible, only 15 isolates displayed wild-type PBP genotype 2-2-2, 2-2-3, or 2-2-71. Isolates in the major RFEL clusters, clusters VIII and VI, displayed PBP genotypes 1-1-1 and (26)-3-30, respectively. The *pbp1a* gene of the last PBP cluster could not be amplified from 5 of 12 strains. In addition, genetic analysis of tetracycline resistance genes *tet*(M) and *tet*(O) revealed that the *tet*(M) gene was exclusively observed in 77 of the 84 pneumococcal isolates (91%). Interestingly, 13 *tet*(M)-containing strains were fully susceptible to tetracycline. Finally, the erythromycin resistance gene *erm*(B) was present in 70 of the 84 pneumococcal isolates (83%). Seven of the *erm*(B)-containing strains also harbored the *mef*(E) gene. In the remaining 14 strains, *erm*(B), *mef*(A), and *mef*(E) were absent. Ten *erm*(B)-containing isolates and one *erm*(B)- and *mef*(E)-containing isolate were erythromycin susceptible (data not shown).

DISCUSSION

To determine the serotype distribution and theoretical rate of coverage of the new pneumococcal conjugate vaccines in Vietnam, we investigated 84 pneumococcal strains isolated from children under 5 years of age who visited several outpatient clinics in Hanoi with acute respiratory tract infections during the period from September 1997 to November 1999. Serotypes 23F (32%), 19F (21%), 6B (13%), and 14 (10%) were predominantly observed. The theoretical rate of coverage of the seven-valent conjugate vaccine was 80%. Since several studies have demonstrated a cross-reactive effect against serotype 6A, we assume a cumulative rate of coverage of the conjugate vaccine of 83% (7, 32). These data are comparable to those found in studies conducted in Europe and the United States, where rates of coverage of 60 to 80% are expected (17, 19, 21, 31, 33, 39).

A high rate of drug resistance was observed among the pneumococcal isolates. Ninety-six percent of the isolates were (intermediate) resistant to penicillin, co-trimoxazole, tetracycline, erythromycin, or cefotaxime. Seventy-eight percent of the isolates were resistant to three or more classes of antibiotics. This suggests a major problem with respect to treatment of pneumococcal infections with these antibiotics.

The epidemiological behavior of the pneumococcal strains isolated from the Vietnamese children was investigated by RFEL analysis. Among the 84 strains, 35 different genotypes were observed, of which 12 genotypes represented eight clusters with 61 strains. This indicates that 73% of the strains were recently transmitted. Ninety-three percent of the clustered isolates displayed capsular serotypes, which are covered by the seven-valent conjugate vaccine. The two largest clusters, clusters VI and VIII, containing 12 and 24 isolates, respectively, were identical to international clones Taiwan 19F-14 and Spain 23F-1, respectively. The remaining clusters, with 42% of the recently transmitted strains, and all strains with unique genotypes were considered to be Vietnam specific, as they were not previously observed for isolates from the 16 countries present in our database. These data indicate that strains of nationally and internationally spreading genotypes make a significant contribution to nasopharyngeal carriage and, consequently, respiratory tract infections among children in Hanoi. The four largest clusters, with 53 strains, i.e., 87% of the disseminating isolates, were multidrug resistant. This indicates a significant transmission of multidrug-resistant pneumococci among children in Hanoi.

Because of the high percentage of resistance to penicillin, erythromycin, and tetracycline among the collection of isolates, we analyzed the corresponding resistance genes in detail. Since the degree of genetic heterogeneity of *pbp* genes in both penicillin-susceptible and penicillin-nonsusceptible isolates is high, a high degree of genetic plasticity irrespective of the penicillin resistance phenotype is suggested. This observation, which is in contrast to previous data, which showed that penicillin-susceptible pneumococci display a limited number of PBP genotypes (36), is in line with recent findings for pneumococcal isolates from Greece (4; Sluijter, unpublished). PBP genotype 1-1-1, which has been predominantly observed in various pneumococcal clones, including pandemic clones Spain 23F-1 and France 9V-3 (4), was also observed in Vietnamese cluster VIII,

which represents clone Spain 23F-1. The second largest, Vietnam-specific cluster, cluster VI, primarily displayed PBP genotype 26-3-30.

Genetic analysis of tetracycline resistance genes *tet*(M) and *tet*(O) revealed the *tet*(M) gene in 91% of the Vietnamese isolates. This is in line with observations made by Luna and Roberts (24), who have demonstrated the presence of the *tet*(M) gene in 90% of the tetracycline-resistant strains. However, in the latter study, an additional 10% of the strains contained the *tet*(O) gene. Thirteen *tet*(M)-containing strains were fully susceptible to tetracycline, suggesting the presence of a nonfunctional tetracycline resistance gene. A similar observation has previously been reported by Doherty et al. (10).

The erythromycin resistance gene *erm*(B) was present in 82% of the pneumococcal isolates, and 10% of the *erm*(B)-containing strains also harbored the *mef*(E) gene. This is in contrast to recent observations made by Reinert and coworkers (29), who have observed almost equal distributions of *erm*(B) (43%) and *mef*(E) (56%) genes among erythromycin-resistant isolates. Ten *erm*(B)-containing isolates and one *erm*(B)- and *mef*(E)-containing isolate were erythromycin susceptible. To our knowledge, this is the first study to describe the occurrence of nonfunctional erythromycin resistance genes in pneumococci.

When our data are compared with those from a recent study of Lee et al. (23), who have investigated pneumococcal carriage in healthy children in South Korea, we noticed slight differences in the serotype distributions. In both studies the three most common serogroups were 6, 23, and 19. However, the rate of carriage of serotype 23F was twice as high in the Vietnamese children with upper respiratory tract infections (30%) as in healthy carriers in South Korea (15%), whereas the rate of carriage of serogroup 6 was significantly higher in the healthy children in South Korea. In both studies, serogroup 23 isolates represented international clone Taiwan 23F. Assuming that the serotype distributions among children in Vietnam and South Korea are comparable, we hypothesize that clone Taiwan 23F is more often correlated with respiratory disease, whereas serogroup 6 is more frequently correlated with carriage. To strengthen this hypothesis, comparative epidemiological studies demonstrating a significant correlation between pneumococcal carriage and respiratory disease are required.

In conclusion, our study demonstrated a high theoretical rate of coverage of the seven-valent conjugate vaccine against pneumococcal carriage strains isolated from children with acute respiratory tract infections in Hanoi. Moreover, the genotypes transmitted the most frequently were covered by the conjugate vaccine. Finally, 78% of the pneumococcal isolates are multidrug resistant, and 92% of these multidrug-resistant isolates are theoretically covered by the seven-valent conjugate vaccine. Our data suggest that the children in Vietnam will benefit from implementation of vaccination with the pneumococcal conjugate vaccine.

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